

Toxic and bloom-forming Baltic Sea cyanobacteria under changing environmental conditions

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ACADEMIC DISSERTATION

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Cover image: Satellite image of the cyanobacterial bloom in the Baltic Sea on 24th of July 2017 combined with transcriptional profile of *N. spumigena* UHCC 0039 cyanobacterium. Satellite image: Finnish Environment Institute, SYKE.

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List of original publications

This thesis is based on the following publications and manuscripts:

- I **Teikari JE***, Hou S*, Wahlsten M, Hess WR, Sivonen K (2018) Comparative genomics of the Baltic Sea toxic cyanobacteria *Nodularia spumigena* UHCC 0039 and its response to varying salinity. *Frontiers in Microbiology*, 9: 356.
- II **Teikari JE***, Hou S*, Wahlsten M, Hess WR, Sivonen K (2018) Insight into the genome and brackish water adaptation strategies of toxic and bloom-forming Baltic Sea *Dolichospermum* sp. UHCC 0315. To be submitted.
- III **Teikari JE**, Österholm J, Kopf M, Battchikova N, Wahlsten M, Aro E-M, Hess WR, Sivonen K (2015) Transcriptomic and proteomic profiling of *Anabaena* sp. strain 90 under inorganic phosphorus stress. *Applied and Environmental Microbiology*. 81(15): 5212–5222.
- IV **Teikari JE**, Fewer DP, Shrestha R, Hou S, Leikoski N, Mäkelä M, Simojoki A, Hess WR, Sivonen K (2018) Strains of the toxic and bloom-forming *Nodularia spumigena* (cyanobacteria) can degrade methylphosphonate and release methane. *ISME Journal*. In press. doi: 10.1038/s41396-018-0056-6

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Author's contribution

- I Jonna Teikari designed the study, executed the experimental work, participated in data analysis and interpretation, and wrote the first draft of the manuscript.
- II Jonna Teikari designed the study, executed the experimental work, participated in data analysis and interpretation, and wrote the first draft of the manuscript.
- III Jonna Teikari was involved in designing the study, carried out the transcriptomic experiment and data analysis, participated in the proteomics study, and wrote the first draft of the manuscript.
- IV Jonna Teikari designed the study, carried out the research and data analysis, and wrote the first draft of the manuscript.

Abbreviations

2APn	Aminoethylphosphonate
16S rRNA	16S ribosomal RNA
σ factor	Sigma factor
AAI	Average amino acid identity
ANI	Average nucleotide identity
APA	Alkaline phosphatases
<i>arsB</i>	Arsenite efflux protein gene
cDNA	Complementary DNA
CO ₂	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
DOP	Dissolved organic phosphorus
EPn	Ethylphosphonate
GC	Gas chromatography
LC	Liquid chromatography
<i>mcy</i>	Microcystin synthetase gene cluster
MPn	Methylphosphonate
MS	Mass spectrometry
N ₂	Nitrogen gas
nc-RNA	non-coding RNA
<i>nda</i>	Nodularin synthetase gene cluster
NRPS	Nonribosomal peptide synthetase
PacBio	Pacific Biosciences
<i>pepM</i>	Phosphoenolpyruvate phosphomutase gene
P _i	Inorganic phosphorus
<i>phn</i>	Phosphonate gene cluster
PstABCS	High-affinity phosphate transporter
PSU	Practical salinity unit
RNA-seq	RNA sequencing
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
SMRT sequencing	Single molecule, real-time sequencing
SNP	Single nucleotide polymorphism
sp.	Species
UHCC	University of Helsinki Cyanobacteria Culture Collection

Abstract

The Baltic Sea is a shallow brackish water ecosystem. It is naturally highly prone to eutrophication, and massive cyanobacterial blooms are an annual phenomenon in this region. Cyanobacterial blooms in the Baltic Sea are toxic to humans and animals, and cause economical losses and harm for recreational users. Almost all cyanobacteria are photoautotrophic organisms, and many bloom-forming Baltic Sea cyanobacteria can additionally fix atmospheric nitrogen. Inorganic phosphorus is usually the first and most important growth-limiting factor for these cyanobacteria. Monitoring the external phosphorus inflow is strictly implemented by the coastal states, but uneven point load still occurs. In addition, a heavy and long-term phosphorus load has resulted in substantial phosphorus reservoirs in the sediments. Sedimented phosphorus can be circulated back to the waterbody, where it becomes available again for microbes such as cyanobacteria. Cyanobacterial blooms in the Baltic Sea are dominated by *Aphanizomenon* sp., *Dolichospermum* sp., and *Nodularia spumigena*, of which *Dolichospermum* sp. and *N. spumigena* can produce toxins. Due to their evolutionary history, *Dolichospermum* sp. is more abundant in the less saline coastal regions, whereas *N. spumigena* dominates in the open sea. This work studied the effects of changing environmental conditions on the distribution and niche adaptation strategies of toxic and bloom-forming Baltic Sea cyanobacteria using state-of-the-art sequencing and molecular biology methods.

Climate change models have predicted that the salinity of the Baltic Sea will possibly decline in the future, and thus the behavior of *Dolichospermum* sp. and *N. spumigena* originating from freshwater and brackish water environments, respectively, was studied in unfavorable salinities. In this study, comparative genomic analysis showed that *Dolichospermum* sp. UHCC 0315 has high synteny between its freshwater counterparts, and possibly therefore the strain was unable to proliferate in moderate salinities. Salt addition induced massive transcriptional shifts, especially within the photosynthesis and nitrogen-fixing pathways. Moreover, moderate salinity increased the production of microcystins and triggered the oxidative stress response. On the contrary, *N. spumigena* UHCC 0039 thrived in higher salinities, and its growth and metabolism were hindered by freshwater. Unique sigma factors and an elevated number of transposases were identified in the genome of *N. spumigena* UHCC 0039, suggesting a high genetic capacity to adapt to changing salinities and brackish water conditions.

The growth and metabolism of *Dolichospermum* sp. and *N. spumigena* were arrested under limited availability of inorganic phosphorus. Both strains upregulated the genes in the *pho* regulon, indicating that these genes may be used for determining the phosphorus status of cyanobacterial blooms. Bioinformatics and PCR based analyses showed that all studied strains of *N.*

spumigena from the Baltic Sea carried the *phnC-M* gene cluster, which is responsible for the transport and utilization of the chemically highly stable phosphonates. Furthermore, laboratory studies demonstrated that naturally produced phosphonates were an additional phosphorus source for *N. spumigena* cyanobacteria, and produced a competitive advantage in phosphorus-limited conditions. However, methane, an organic remnant of methylphosphonate, was not utilized by the studied cyanobacteria, and it was released to the gaseous environment. Massive blooms of *N. spumigena* cyanobacteria may thus explain the elevated summertime methane concentration in the Baltic Sea. The results presented in this thesis suggest that cyanobacterial blooms will continue to appear in the future if sufficient amount of phosphorus is present but community composition may shift towards freshwater species as a consequence of climate change.

Tiivistelmä

Itämeri on matala murtovesiallas ja se on luontaisesti erittäin altis rehevöitymiselle. Massiiviset syanobakteeri- eli sinileväkukinnat ovatkin jokakesäinen ilmiö Suomenlahdella, Saaristomerellä ja Itämeren pääaltaalla. Kukinnat ovat myrkyllisiä ihmiselle ja muille nisäkkäille ja aiheuttavat siten paljon haittaa veden virkistyskäyttäjille. Kaikki syanobakteerit ovat yhteyttäviä organismeja. Lisäksi Itämerellä kukintoja muodostavat lajit kykenevät sitomaan ilmakehän typpeä, minkä seurauksena epäorgaanisen fosforin saatavuus toimii tyypillisesti tärkeimpänä syanobakteerien kasvua rajoittavana tekijänä. Itämerta ympäröivät valtiot valvovatkin nykyään tiukasti maalta tulevia epäorgaanisia fosforipäästöjä (= ulkoinen kuormitus), mutta satunnaisia pistekuormituksia tapahtuu edelleen. Pitkään jatkunut raskas fosforikuorma on kuitenkin yhä sitoutuneena pohjan sedimentteihin, mistä se vapautuu hapettomilta pohja-alueilta vähitellen takaisin vesipatsaaseen (= sisäinen kuormitus). Itämeren syanobakteerikukinnat koostuvat *Aphanizomenon* sp., *Dolichospermum* sp. ja *Nodularia spumigena* -lajeista, joista kaksi jälkimmäistä tuottavat myrkyjä. Evoluutiivisen historiansa vuoksi *Dolichospermum* sp. esiintyy runsaammin vähäsuolaisemmillä rannikkovesialueilla, kun taas *N. spumigena* vallitsee suolaisemmalla avomerellä. Tässä työssä Itämeren myrkyllisten ja kukintoja muodostavien syanobakteerien esiintymistä ja kykyä sopeutua ilmastonmuutoksesta ja ihmisen toiminnan seurauksesta aiheutuvaan muuttuvaan ympäristöön on arvioitu nykyaikaisten sekvensointi- ja molekyylibiologisten työkalujen avulla.

Koska Itämeren suolapitoisuuden on ennustettu madaltuvan ilmastonmuutoksen seurauksena, tämän työn ensimmäisessä osassa selvitettiin, miten makean veden *Dolichospermum* sp. ja murtoveden *N. spumigena* käyttäytyvät epäsuotuisissa suolapitoisuuksissa. Laboratoriokokeet osoittivat, että *Dolichospermum* sp. UHCC 0315 on geneettisiltä ominaisuuksiltaan hyvin samankaltainen makean veden sukulaistensa kanssa, eikä se kykene kasvamaan korkeissa suolapitoisuuksissa. Korkeat suolapitoisuudet aiheuttivat mittavaa transkriptomin uudelleenjärjestäytymistä, mikä näkyi erityisesti fotosynteesi- ja typensidontakoneistojen toiminnan tukahduttamisena. Lisäksi korkea suolapitoisuus lisäsi myrkyntuotantoa sekä lipidien uudelleenjärjestäytymistä, mikä viittaa siihen, että korkea suolapitoisuus laukaisi soluissa oksidatiivisen stressireaktion. Päinvastoin kuin *Dolichospermum* sp., *N. spumigena* UHCC 0039 kasvoi parhaiten korkeammissa suolapitoisuuksissa, ja matalammat suolapitoisuudet aiheuttivat metabolian hidastumista. *N. spumigena* UHCC 0039 -syanobakteerin genomisekvenssistä pystyttiin tunnistamaan ainutlaatuisia sigma-faktoreita ja suuri määrä liikkuvia geneettisiä elementtejä, joilla on todennäköisesti tärkeä rooli *N. spumigenan* sopeutumiskyvylle vaihteleviin suolapitoisuuksiin.

Sekä *Dolichospermum* sp. että *N. spumigena* -lajien kasvu ja metabolia hidastuivat kokeissa, joissa fosforin määrää kasvualustassa oli rajoitettu. Molemmat kannat ilmensivät myös voimakkaasti *pho*-säättelyalueen geenejä, joita voidaan hyödyntää määritettäessä fosforin määrää syanobakteerikukintojen aikana. Bioinformatiikan ja PCR-analyysien avulla kaikilta tutkituilta Itämeren *N. spumigena* -kannoilta löydettiin *phnC-M*-geenialue, joka on vastuussa kemiallisesti hyvin stabiilien fosfonaattimolekyylien kuljettamisesta ja pilkkomisesta. Laboratoriokokeet osoittivat, että luonnollisesti tuotetut fosfonaatit toimivat vaihtoehtoisena fosforinlähteenä *N. spumigena* -syanobakteereille ja tarjoavat lajille kilpailuedun alueilla, joilla epäorgaanisen fosforin saatavuus on niukkaa. *N. spumigena* ei kuitenkaan hyödyntänyt metyylifosfonaatista vapautuvaa orgaanista hiilimolekyyliä, metaania, ja metaanipitoisuuden määrä ympäröivässä kaasutilassa kasvoi. Massiivisilla *N. spumigena* -kukinnoilla saattaakin olla yhteys Itämeressä kesäisin havaittuihin kohonneisiin metaanipitoisuuksiin. Tässä työssä esitettyjen tulosten pohjalta voidaan päätellä, että syanobakteerikukinnat eivät katoa Itämerestä tulevaisuudessakaan mikäli kokonaisfosforin määrää ei saada pienennettyä, mutta kukintojen lajikoostumus saattaa muuttua ilmastonmuutoksen seurauksena.

1 Introduction

The Baltic Sea

The Baltic Sea is a small and shallow multi-basin brackish water ecosystem possessing a unique salinity gradient running from freshwater coastal regions to a nearly marine environment in the Kattegat. The horizontal salinity gradient is a consequence of riverine freshwater inflow from the drainage area and saline water pulses via the Danish Straits (Zillén *et al.*, 2008). Due to shallow and narrow sills to the North Sea, the water retention time in the Baltic Sea is over 30 years (Döös *et al.*, 2004). The inflow of lighter surface freshwater and denser saline water, in turn, hinders water turnover between the bottom and surface, causing a stratified vertical water column with a halocline at a depth of 60–80 m (Carstensen *et al.*, 2014). Stagnation of the bottom water makes the ecosystem naturally extremely prone to oxygen deficiency, i.e. hypoxia.

The salinity gradient of the Baltic Sea induces a unique species distribution in the area. Several microbial brackish water specialists have evolved, and there is high microbial species diversity in the brackish water (3.5–16 PSU) region (Herlemann *et al.*, 2011; Telesh *et al.*, 2013; Celepeli *et al.*, 2017). However, multicellular species occur in fewer numbers and are genetically less diverse compared to the North Atlantic (Johannesson and André, 2006). Based on climate change models, the average salinity of the Baltic Sea is predicted to decrease due to increased precipitation and riverine runoff (Kjellström and Ruosteenoja, 2007; Vuorinen *et al.*, 2015). A decrease in salinity may directly affect the distribution of the prevailing species, thus increasing the abundance of freshwater specialists and causing southward migration of marine species (HELCOM, 2007; Vuorinen *et al.*, 2015). Increased riverine runoff may additionally accelerate nutrient inflow from the catchment area, favoring the occurrence of bloom-forming primary producers (HELCOM, 2007).

The Baltic Sea is highly influenced by the anthropogenic activity of 85 million people living around the drainage basins. Increased agricultural, sewage, and industrial-based phosphorus and nitrogen loads have enhanced primary production and eutrophication in this area (HELCOM, 2009). Elevated nitrogen availability increases spring blooms of diatoms, whereas phosphorus is crucial for the formation of diazotrophic cyanobacterial blooms in later summer (Vahtera *et al.*, 2007). The release of sedimented phosphorus to the water column (Lukkari *et al.*, 2008) further enhances the formation of cyanobacterial blooms (Funkey *et al.*, 2014). International management of nutrient inflow has improved the status of the coastal zones (Elmgren *et al.*, 2015), although the Baltic Sea is still highly vulnerable to eutrophication due to the phosphorus reservoirs in the sediments.

Cyanobacterial blooms in the Baltic Sea

Cyanobacteria are prokaryotic organisms harboring protein complexes involved in oxygenic photosynthesis, very similar to those found in algae and higher plants. They thrive in all light-exposed habitats including aquatic (marine, brackish, and freshwater) and terrestrial (e.g. soil, rocks, and lichen symbioses) environments (Whitton and Potts, 2000; Adams *et al.*, 2012). Cyanobacteria display enormous morphological and physiological diversity: they can be unicellular, colonial, or filamentous, their vegetative cells can develop into nitrogen-fixing heterocysts, and they can possess resting cells, i.e. akinetes, motile filaments, or hormogonia (Castenholz, 2001; Meeks *et al.*, 2002). Cyanobacteria employ a multitude of strategies to enhance their fitness in aquatic environments, such as gas vacuoles for maintaining favorable depth in the water column, siderophores for effective iron uptake, and high-affinity phosphate transporters for sufficient acquisition of inorganic phosphorus (Murphy *et al.*, 1976; Su *et al.*, 2007; Penn *et al.*, 2014; Rudolf *et al.*, 2015; Santos-Beneit, 2015). Cyanobacteria are well known for their capacity to produce a wide variety of natural bioactive compounds (Welker and von Döhren, 2006). Among these natural products, toxins are the most widely studied group due to harming eukaryotes (Sivonen and Jones, 1999; Codd *et al.*, 2017).

Cyanobacteria may form massive blooms mostly in eutrophic water bodies (Paerl and Huismann, 2008; Conley *et al.*, 2009; Paerl and Paul, 2012). The nutrient-enriched Baltic Sea thus serves as an ideal environment for the growth of cyanobacteria during the warm summer months, and annual blooms have caused cyanobacteria to be recognized as an issue of public concern in this area. Blooming cyanobacteria, *Nodularia spumigena*, *Aphanizomenon* sp., and *Dolichospermum* sp. (former name *Anabaena* sp.), are buoyant and form surface scums causing physical and odorous nuisance for recreational users of the Baltic Sea (Sivonen *et al.*, 2007; Kaloudis *et al.*, 2017). However, the presence of cyanobacterial toxins causing animal and human poisoning is the most severe problem (Chen *et al.*, 2013). *N. spumigena* (Blackburn *et al.*, 1996; Akcaalan *et al.*, 2009; Popin *et al.*, 2016) and *Dolichospermum* sp. (Rippka *et al.*, 2015) blooms are ubiquitous in eutrophic fresh and brackish water ecosystems across the world, and toxic strains have also frequently been found from cyanobacterial blooms in the Baltic Proper and the Gulf of Finland (Sivonen *et al.*, 1989; Halinen *et al.*, 2007). Generally, *Dolichospermum* sp. is a freshwater cyanobacterium, whereas *N. spumigena* is a brackish water species and has found to be always toxic in the environment (Laamanen *et al.*, 2001; Lyra *et al.*, 2005). Due to morphological and physiological similarities between *Dolichospermum* sp. and *N. spumigena*, salinity has been suggested to be the most important abiotic factor controlling their distribution in the Baltic Sea (Lehtimäki *et al.*, 1997; Mazur-Marzec *et al.*, 2005; Brutemark *et al.*, 2015).

The frequency of *Dolichospermum*, which originates from freshwater, increases in less saline regions and estuaries, whereas *N. spumigena* outcompetes it in higher salinity regions (Kahru *et al.*, 2000; Celepli *et al.*, 2017).

Toxins produced by *N. spumigena* and *Dolichospermum* sp. strains are called nodularins and microcystins, respectively (Sivonen *et al.*, 1989; Halinen *et al.*, 2007). Due to the similarities between the gene clusters and mechanism of action, the biosynthesis of nodularin and microcystin is assumed to have a common evolutionary origin (Moffitt and Neilan, 2004; Rantala *et al.*, 2004). Both of these cyclic peptides, synthesized by a hybrid nonribosomal peptide synthetase (NRPS)/polyketide synthase, are hepatotoxins inhibiting protein phosphatase of types 1, 2A, and 3–5 in the liver cells (Moffitt and Neilan 2004; Dittmann *et al.*, 2012; Catherine *et al.*, 2017). Studies on whether nodularins and microcystins have allelopathic effects on other living organisms, such as phyto- and zooplankton, aquatic plants or animals, have produced contradictory results (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Engström-Öst *et al.*, 2015; Catherine *et al.*, 2017; Wang *et al.*, 2017). In addition to toxins, *N. spumigena* and *Dolichospermum* produce a large variety of other biologically active natural products (Sivonen *et al.*, 2010; Wang *et al.*, 2012; Fewer *et al.*, 2013; Voß *et al.*, 2013; Vestola *et al.*, 2014), which may also contribute to the allelopathy of these cyanobacteria. Cyanobacterial blooms may comprise both toxic and non-toxic species and strains. Toxic and non-toxic strains are indistinguishable based on their morphology alone (Salmaso *et al.*, 2017), and thus, without the usage of validated toxin detection methods based on chemistry and biochemistry (Kós *et al.*, 1995; Sangolkar *et al.*, 2006), the presence of *N. spumigena* and/or *Dolichospermum* sp. always entails a concrete health risk for humans and other mammals.

Role of aquatic cyanobacteria in the biogeochemical cycles

Photosynthetic cyanobacteria contributed to the formation of an oxygen-rich atmosphere on Earth billions of years ago, and during the course of evolution, endosymbiosis between protists and cyanobacteria resulted in the formation of membrane-bound chloroplasts found in algae and higher plants (Nowack and Melkonian, 2010). Due to oxygenic photosynthesis, cyanobacteria are major contributors to global carbon fluxes, comprising at least 25% of the primary production worldwide (Bullerjahn and Post, 2014). In addition, diazotrophic cyanobacteria increase the pool of bioavailable nitrogen by fixing atmospheric nitrogen gas (N₂) (Larsson *et al.*, 2001). In the Baltic Sea, vegetative cells of the bloom-forming genera *N. spumigena*, *Aphanizomenon flos-aquae*, and *Dolichospermum* sp. can develop into nitrogen-fixing cells, i.e.

heterocysts (Fay *et al.*, 1968; Castenholz, 2001). Heterocysts lack an oxygenic photosystem, which protects the oxygen-sensitive nitrogenase, the key enzyme needed for nitrogen fixation (Haselkorn, 1978), and they alleviate nitrogen deficiency in vegetative cells under nitrogen scavenging conditions (Kumar *et al.*, 2010). However, excess organic nitrogen leaks into the surrounding environment, becoming available for other organisms such as cyanobacteria-associated bacteria and picocyanobacteria (Larsson *et al.*, 2001, Stal *et al.*, 2003, Ploug *et al.*, 2011; Coloma *et al.*, 2016). The total amount of nitrogen input by diazotrophic cyanobacteria to the Baltic Sea is estimated to be at the same level as the riverine load (Larsson *et al.*, 2001), whereas they produce 20–40% of the total carbon biomass during summer. Cyanobacteria are thus important players in carbon and nitrogen fluxes, constructing affluent and nutrient-rich (micro)environments in aquatic ecosystems.

Diazotrophic cyanobacteria gain an advantage in nitrogen limited systems over organisms that do not possess the capacity to fix dissolved N₂ gas. Therefore, phosphorus is usually the most important nutrient alleviating nutrient limitation of growth for diazotrophic cyanobacteria (Graneli *et al.*, 1990, Kononen *et al.*, 1996, Karl *et al.*, 2001, Sundareshwar *et al.*, 2003, Moisander *et al.*, 2003; 2007; 2012, Dyhrman and Haley, 2006). Due to its crucial role, input of inorganic phosphorus (P_i) from the land has been carefully controlled and monitored in the Baltic Sea area (Svendsen *et al.*, 2015) to hinder cyanobacterial growth and eutrophication pressure in the Baltic Sea (Conley *et al.*, 2009). Nonetheless, uneven point loads from coastal areas and increased release of P_i from bottom areas are suggested to influence the intensity of future cyanobacterial blooms (Kahru *et al.*, 2000).

Inorganic phosphorus at the oxidation state of +5 (P_i; PO₄³⁻) is generally considered the most preferable form of phosphorus for cyanobacteria. Inorganic phosphorus is taken up via a PstABCS transporter, the expression of which is regulated by the sensitive two-component regulatory system PhoR/PhoB/PhoU (*pho* regulon) (Figure 1) (Yamada *et al.*, 1989; Su *et al.*, 2007; Santos-Beneit, 2015). To cope better under P_i scarcity, enzymes called alkaline phosphatases (APA), which liberate P_i from the organophosphates, are widely distributed within the Cyanobacteria phylum (Sebastian and Ammerman, 2009). The APA synthesizing gene is also located under the *pho* regulon activated by environmental P_i scarcity (Li *et al.*, 1998; Nausch, 1998; Van Wambeke *et al.*, 2002). In addition, certain cyanobacteria have access to another pool of phosphorus molecules at the oxidation state of +3 (phosphites, PO₃³⁻ and phosphonates, C–PO(OR)₂) (Van Mooy *et al.*, 2015). To utilize phosphites and phosphonates, specific enzyme complexes are needed to release P_i from the organic backbone (Wanner, 1994; White and Metcalf, 2004; Adams *et al.*, 2008; Ilikchyan *et al.*, 2009; Martinez *et al.*, 2010; Villarreal-Chiu *et al.*, 2012).

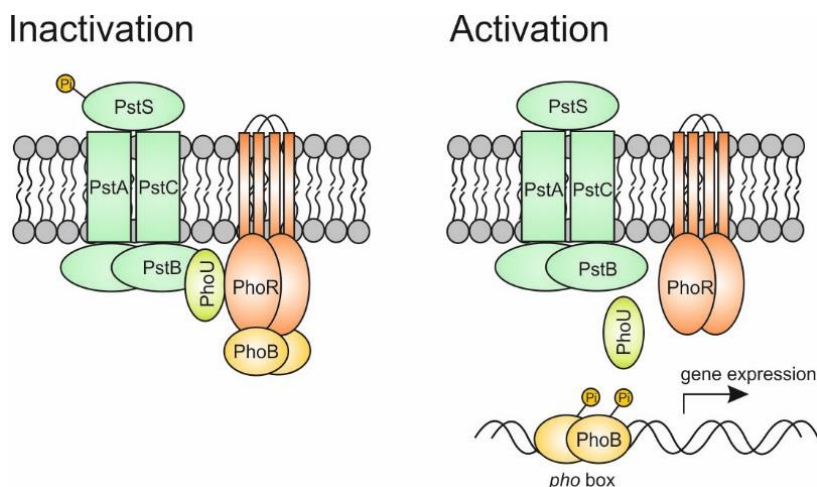


Figure 1 Activation and inactivation of *pho* regulon. Lack of external inorganic phosphorus triggers morphological changes in the high-affinity phosphate transporter system (PstABCS) embedded in the cell membrane, leading to autophosphorylation of PhoB. Phosphorylated PhoB binds to the *pho* box located upstream of the promoter site and activates the downstream region of DNA. Figure modified from McGrath *et al.*, 2013.

Phosphonates are an enigmatic pool of phosphorus molecules that have either natural (e.g. methylphosphonate, 2-aminomethylphosphonate) or anthropogenic (used in numerous industrial applications e.g. pesticides and detergents) origin (Kononova and Nesmeyanova, 2002; Dyhrman *et al.*, 2009; Metcalf *et al.*, 2012, Rott *et al.*, 2018). A nearly unbreakable C-P bond makes them extremely stable in nature (Karl, 2014), and due to their inert structure, phosphonates have replaced P_i in many industrial applications to reduce the release of P_i to the environment. In nature, organisms, mainly bacteria carrying the *pepM* gene, are capable of producing phosphonates for use as structural components in cell wall phospholipids and polysaccharides (Bowman *et al.*, 1988, Villarreal-Chiu *et al.*, 2012), but also in antibiotic molecules (Metcalf and van der Donk, 2009). Phosphonates represent approximately 5–10% of the total dissolved organic phosphorus pool (DOP) in marine ecosystems (Young *et al.*, 2010), but due to restrictions of determination methods, the deeper structure of the phosphonate pool is poorly characterized (Skeff *et al.*, 2015). Interestingly, phosphonates have the potential to serve as an alternative phosphorus source for Baltic Sea cyanobacteria, as the genome of the Baltic Sea isolate *N. spumigena* CCY 9414 was found to encode the C-P lyase pathway (Voß *et al.*, 2013). This multi-enzyme complex is capable of phosphonate transport and C-P cleavage (*phnC-P*), and is highly similar to those found in the genomes of heterotrophic bacteria (Metcalf and Wanner, 1991; White and Metcalf, 2004) along with *Trichodesmium erythraeum* IMS101 (Dyhrmann *et al.*, 2006) and *Synechococcus* sp. JA-2-3Ba(2-13) (Adams *et*

al., 2008). Both *Trichodesmium erythraeum* IMS101 and *Synechococcus* sp. JA-2-3Ba(2-13) can grow in medium containing phosphonates as the sole source of phosphorus (Dyhrman *et al.*, 2006; Adams *et al.*, 2008). However, variations in the amino acid compositions of PhnD proteins affect their dissociation constants, and the ability to utilize phosphonate may vary between species and even between strains (Forlani *et al.*, 2008; Feingersch *et al.*, 2012).

Recent studies have linked phosphonate producers and utilizers to aerobic methane release in the epipelagic zones of marine ecosystems (Karl *et al.*, 2008; Gomez-Garcia *et al.*, 2011, Repeta *et al.*, 2016). This may partially explain the oceanic methane paradox, where the concentration of methane in surface water is above the atmospheric equilibrium. Higher methane concentrations are also detected in the surface water of the Baltic Sea in summer, but the source has been elusive to date (Bange *et al.*, 1994; 1998).

Stress responses of cyanobacteria

Salinity fluctuation, high irradiance, and phosphorus and carbon dioxide (CO₂) deficiency represent major abiotic stressors that nitrogen-fixing cyanobacteria encounter in the Baltic Sea. However, due to their large gene pools (Voß *et al.*, 2013) and dynamic genomes (Kopf *et al.*, 2014), these cyanobacteria are capable of rapid adaptation to a new environment by shifting their transcriptome and evoking stress responses. Changes in gene expression may occur within minutes after confronting a new environment (Pade and Hagemann, 2014), and thus comparative transcriptomics is an efficient tool for studying varying environmental conditions (see next chapter). Stress responses are divided into two groups: general and stress-specific responses. In cyanobacteria, general stress responses by altering the expression of genes related to photosynthesis, light harvesting complexes, transcription, translation, and central metabolism (Dyhrman and Haley, 2006; Allakhverdiev and Murata, 2008; Billis *et al.*, 2014; Rai *et al.*, 2013; Al-Hosani *et al.*, 2015), ultimately result in hindered growth (Rapala *et al.*, 1997; Marin *et al.*, 2004; Repka *et al.*, 2004). Sigma factors (σ factors) have an essential role in initiating stress responses in cells. They bind to the RNA polymerase (core enzyme) so that the polymerase complex (holoenzyme) can recognize and bind to DNA to initiate transcription (Paget and Helmann, 2003). In response to environmental changes, a σ factor can be replaced by another type of σ factor, causing changes in the structure and binding capacity of the RNA polymerase, in turn, inducing or hindering gene expression. The σ^{70} family in cyanobacteria is divided into three phylogenetic subgroups (I–III), which are structurally and functionally diverse, recognizing various promoter regions (Imamura and

Asayama, 2009). For instance, in the model cyanobacterium *Synechocystis* sp. PCC 6803, group 1 contains only SigA, which is essential for cellular viability, and group 2 is similar to group one comprising SigB–SigE, whereas group III σ factors are essential for stress responses (SigF–I) (Marin *et al.*, 2004; Singh *et al.*, 2006; Tuominen *et al.*, 2006; Nikkinen *et al.*, 2012; Antal *et al.*, 2016). Heat shock, darkness, and nutrient deficiency all have an impact on the expression of the σ factor pool and fine-tuning the expression of σ factors plays an important role in initiating the reconstruction of the transcriptional profile.

Alongside general responses, several stress-specific responses, during which only a minor set of genes is activated, play an important role in enhancing cyanobacterial fitness in varying environments. Stress-specific genes provide a competitive advantage for cyanobacteria, and they can be used as markers to evaluate the cellular and environmental status. Because readily available P_i is taken up effectively (Dyhrman and Haley, 2006) and the concentration of P_i in the surface water is usually very low (Nausch *et al.*, 2004; Olofsson *et al.*, 2016), phosphorus-limitation is characteristic for the Baltic Sea cyanobacteria. Phosphorus deficiency hinders the overall metabolism, growth, and toxin production of nitrogen-fixing cyanobacteria (Lehtimäki *et al.*, 1997; Rapala *et al.*, 1997; Repka *et al.*, 2004), although several stress-specific responses have been reported. Activation of the *pho* regulon caused by the lack of P_i in the extracellular environment is the most widely studied stress-specific response (Figure 1). In cyanobacteria, the *pho* regulon covers genes related to the phosphorus cycle, for example, the *phn* gene cluster and alkaline phosphatases, as well as several separate genes, such as genes related to photosynthesis, nitrogen assimilation, and carbon fixation (Su *et al.*, 2007), which complicates the search for suitable marker genes. One commonly used molecular marker for phosphorus deficiency determines the activity of alkaline phosphatases using fluorescence substrates (Li *et al.*, 1998; Nausch, 1998; Van Wambeke *et al.*, 2002). However, the suitability of this particular marker for implying P_i scarcity has received criticism. More recently, in a study of *Crocosphaera*, Pereira *et al.* (2016) suggested a combination of several *pho* regulon genes to monitor the phosphorus status in cells.

Salinity is another important factor controlling cyanobacterial abundance and species distribution in the Baltic Sea (Sivonen *et al.*, 2007; Pade and Hagemann, 2014; Brutemark *et al.*, 2016; Celepli *et al.*, 2017). In the Baltic Sea, cyanobacterial blooms encounter salt water pulses and freshwater riverine inflow, although overall salinity is suggested to decrease (Kjellström and Ruosteenoja, 2007; von Storch *et al.*, 2015; Graham, 2016). In cyanobacteria, similar to other bacteria, strain-specific concentrations of inorganic ions are needed to maintain constant turgor pressure and cellular functioning (Hagemann, 2011). Increasing salinity changes the ion concentration of the cells, and complex acclimation processes are rapidly

induced to protect cellular structure and metabolism (Hagemann and Marin, 1999; Kanesaki *et al.*, 2002; Marin *et al.*, 2002; 2003; 2004; Fulda *et al.*, 2006; Huang *et al.*, 2006; Nikkinen *et al.*, 2012; Qiao *et al.*, 2013). Acclimation processes include ion exchange through the activation/inactivation of existing transporters along with the expression of stress-related genes and the synthesis of compatible solutes. The effects of low salinity on cyanobacteria has been investigated much less (Paper I in this study).

High-throughput DNA sequencing in microbial ecology

The term 'genomics' was coined in 1986 to denote the study and comparison of genomes across species (Kuska, 1998). At that time, the Sanger sequencing technique was in use, providing among others, the first whole-genome sequence of a living organism, *Haemophilus influenzae* (Fleischmann *et al.*, 1995). Sanger sequencing was the method of choice until the early 2000s, when the invention of high-throughput DNA sequencing drastically lowered the cost and duration of genome sequencing (Metzker *et al.*, 2005; Loman *et al.*, 2012). The new technique rapidly increased the number of sequenced bacterial genomes, first with 454 pyrosequencing and later with Illumina chemistry (Metzker, 2010; Loman *et al.*, 2012). However, most of these genomes have only been sequenced to the draft level because newly invented techniques yield short reads and their assembly has turned out to be difficult. The assembly of cyanobacterial genomes has been especially challenging due to their plasticity and the huge number of repetitive elements (Stucken *et al.*, 2010; Voß *et al.*, 2013 Popin *et al.*, 2016), and only 16% out of 731 sequenced cyanobacterial genomes have been closed to date (meaning single DNA molecules without gaps or ambiguous bases) (Figure 2). Nevertheless, improvements in sequencing technologies have provided microbiologists access to microbial phylogenetic diversity, as well as a holistic overview of previously unknown functionality and metabolism (Haft *et al.*, 2015). In addition, the availability of genome sequences for several strains within one species has enabled the calculation of pan- and core genomes, offering valuable information about the presence or absence of certain metabolic pathways, and thereby providing insights into niche adaptation within species (Medini *et al.*, 2008). More recently, mainly through the development of the long-read Pacific Biosciences single molecule real-time sequencing (SMRT) technology (Quail *et al.*, 2012; Koren *et al.*, 2012) and demand for high-quality references, sequencing of whole and high-quality genomes, where each base is determined accurately in the correct order, has increased in popularity. Sequencing the whole genome enables scientists to gain even deeper insights into genomic structures such as accurate genome

size, distinction between chromosomes and plasmids, genome plasticity, single nucleotide polymorphisms (SNPs), and repetitive elements (CRISPR) (López-Pérez *et al.*, 2013; Ekblom and Wolf, 2014; Driscoll *et al.*, 2017). Whole genome analysis also reduces the bias in comparative genomics and transcriptomics studies (López-Pérez *et al.*, 2013; Macrander *et al.*, 2015; Fadeev *et al.*, 2016).

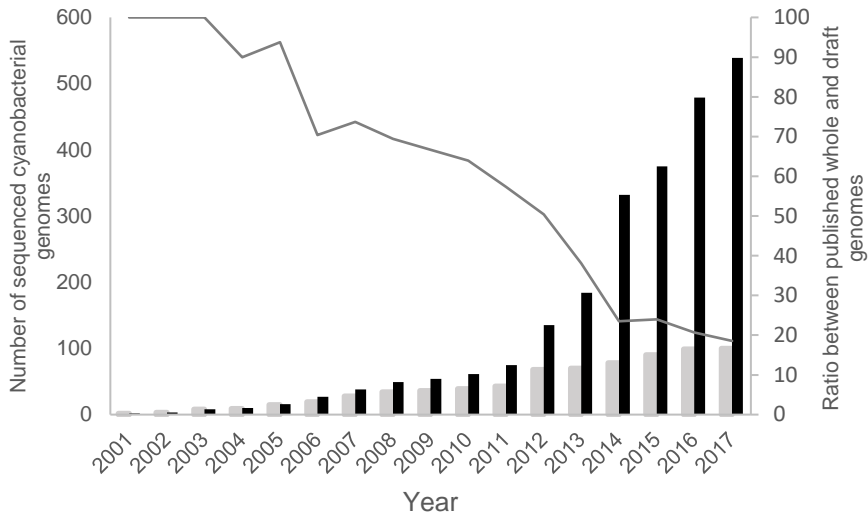


Figure 2 Total number of available cyanobacterial genomes (black bars) and whole genomes (grey bars). Genomes were obtained from NCBI genome database by filtering out all other sequenced genomes but cyanobacteria. Per year numbers were calculated manually. The solid line represents the percentage of whole genomes among all sequenced genomes. The data extends until the end of the year 2017.

Cyanobacterial genomes are highly dynamic entities, and genome sequences only provide a basic framework for their functioning. Importantly, gene expression is regulated by transcription, where RNA is synthesized on DNA, and by post-transcription, where RNA is modified, cleaved, or interfered by non-coding RNA (nc-RNA) (Storz *et al.*, 2011, Kopf *et al.*, 2015). Studies of initial transcriptomes thus add invaluable information to genomic data and provide a holistic approach for building a profile for gene expression along with transcript boundaries, and regions of antisense and nc-RNAs (Sorek and Cossart, 2010; Kopf and Hess, 2014). Measuring the expression of single genes by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) requires a tremendous amount of work, whereas microarrays rely on previously known sequence probes and expensive chip design. Thus, applications of high-throughput DNA sequencing have also revolutionized transcriptome studies. In RNA sequencing (RNA-seq), a pool of complementary DNA (cDNA) molecules are parallel sequenced to gain high

transcriptional coverage, and differentially expressed genes can nowadays be easily determined using bioinformatics pipelines (Wang *et al.*, 2009). High-throughput RNA-seq is overtaking RT-qPCR and microarray techniques in generic gene expression studies owing to improved accuracy, sensitivity, and high dynamic range. Overall, over the past decade, the development of rapid high-throughput DNA sequencing techniques has revolutionized the study of microbial ecology.

Proteomics: quantitative and qualitative analysis of the protein pool

The synthesis of mRNA is only the first step in a long chain of regulatory events resulting in the prevailing phenotype of a cell, and thus the study of proteomes provides a more comprehensive approach to gain knowledge of the cellular state. Many studies have observed weak correlation between mRNA and protein expression levels (Wasinger *et al.*, 2006), and thus proteomics, the method that simultaneously separates and identifies the entire protein pool of an organism, represents a more robust way to study the molecular status of microbial models (Graves and Haystead, 2002). Protein pools are separated either by 2-dimensional gel electrophoresis (2DE) (Wasinger *et al.*, 2006) or by instruments, such as liquid chromatography (LC) and capillary electrophoresis combined directly with mass spectrometry (MS) (Angel *et al.*, 2012). 2DE is an easy and cost-efficient separation method, but its limitations include inappropriate sensitivity for low-level proteins, proteins with extreme isoelectric point or mass, and membrane proteins (Wasinger *et al.*, 2006). Protein identification relies strongly on sequenced genomes and state-of-the-art MS applications, and novel MS techniques have enabled rapid improvements in proteomics in recent years.

2 Study aims

The aim of this study was to gain new information about Baltic Sea cyanobacteria under changing environmental conditions using state-of-the-art sequencing techniques combined with physiological experiments. Climate change and anthropogenic activities cause changes in Baltic Sea hydrology. It is therefore relevant to ask how the abundance and species distribution of cyanobacteria respond to these factors, and what are the key adaptation strategies when cyanobacteria encounter new environments. Two major abiotic factors, change in salinity concentration and inorganic phosphorus limitation, were selected. Moreover, the capacity of Baltic Sea cyanobacteria to utilize anthropogenic and naturally produced phosphonates as an alternative phosphorus source to gain a competitive advantage was tested. The results are regarded and discussed in a wider ecological perspective, providing new information about cyanobacterial ecology in aquatic ecosystems. The detailed aims of the study were:

- to enhance genomic knowledge of bloom-forming and toxic cyanobacteria in the brackish water Baltic Sea (I and II)
- to study the effects of different salinities on the distribution of toxic Baltic Sea cyanobacteria and their transcriptional shifts under unfavorable salt concentrations (I and II)
- to gain insight into the transcriptome and proteome profiles of *Dolichospermum* cyanobacteria under inorganic phosphorus limitation and to detect marker genes for monitoring inorganic phosphorus deficiency (III)
- to study the capacity of *N. spumigena* to utilize naturally produced and anthropogenic-based phosphonates as an alternative phosphorus source (IV)

3 Summary of materials and methods

The cyanobacterial strains used in this study belong to the University of Helsinki Cyanobacteria Collection (HAMBI, UHCC). The taxonomy of the *Anabaena* genus has been reorganized, and the genus is currently divided into two genera: planktonic *Dolichospermum* and benthic *Anabaena* (Wacklin *et al.*, 2009). In addition, the nomenclature of the University of Helsinki Culture Collection was standardized during this study and new strain names have been applied. All strains used in this study, with both new and former names indicated, are listed in Table 1.

Table 1 Cyanobacterial strains used in this study.

Strain	Former name	Origin	Study
<i>Dolichospermum</i> sp.			
<i>Dolichospermum</i> sp. UHCC 0090	<i>Anabaena</i> sp. 90	Lake Vesijärvi	III
<i>Dolichospermum</i> sp. UHCC 0315	<i>Anabaena</i> sp. 315	The Baltic Sea	II
<i>Nodularia</i> sp.			
<i>N. spumigena</i> UHCC 0039	<i>N. spumigena</i> AV1	The Baltic Sea	I, IV
<i>N. spumigena</i> UHCC 0060	<i>N. spumigena</i> HEM	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0041	<i>N. spumigena</i> AV3	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0042	<i>N. spumigena</i> AV33	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0063	<i>N. spumigena</i> AV63	The Baltic Sea	IV
<i>N. spumigena</i> BC Nod-9402		The Baltic Sea	IV
<i>N. spumigena</i> BC Nod-9403		The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0184	<i>N. spumigena</i> Tri 183	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 185	<i>N. spumigena</i> GR7a	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0138	<i>N. spumigena</i> F81	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0143	<i>N. spumigena</i> SR5i	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0083	<i>N. spumigena</i> Fe2a	The Baltic Sea	IV
<i>N. spumigena</i> UHCC 0069	<i>N. spumigena</i> AN13c	The Baltic Sea	IV
<i>N. spumigena</i> NSPI-05		Australia	IV
<i>N. sphaerocarpa</i> UHCC 0048	<i>Nodularia</i> sp. UP16a	The Baltic Sea	IV
<i>N. sphaerocarpa</i> UHCC 0052	<i>Nodularia</i> sp. UP16f	The Baltic Sea	IV
<i>N. sphaerocarpa</i> UHCC 0038	<i>Nodularia</i> sp. HKVV	The Baltic Sea	IV

Environmental samples were collected in August 2016 from a depth of <1 m from the different monitoring stations in the Baltic Sea. Sampling locations and sampling times are presented in Table 2.

Table 2 Environmental sampling.

Sample	Station	Coordinates		Sampling day	Sampling time
		Latitude	Longitude		
1	AALTO_HKI	59°57.90'	25°14.10'	1.8.2016	16:35
2	LL15	59°11.00'	21°44.81'	2.8.2016	6:35
3	LL17	59°02.00'	21°04.77'	2.8.2016	14:05
4	F80	58°00.00'	19°53.81'	3.8.2016	2:05
5	BY15	57°19.20'	20°03.00'	3.8.2016	15:15
6	BY38	57°07.00'	17°40.00'	4.8.2016	12:10
7	F76A	59°05.00'	19°34.80'	5.8.2016	8:45
8	F69	59°47.00'	19°55.80'	5.8.2016	15:45

The methods used in this study are listed in Table 3. Detailed descriptions of these methods are presented in the four articles on which this dissertation is based.

Table 3 Methods used in this study.

Method	Study
Cyanobacterial cultivation	I–IV
Batch culture growth experiment	I–IV
Environmental sampling	IV
DNA and RNA extraction	I–IV
Primer design and PCR amplification	
PCR	II, IV
RT-(q)PCR	IV
DNA sequencing	
Whole genome sequencing by PacBio/Illumina	I–II
RNA sequencing	
SOLiD chemistry	II
Illumina chemistry	I, III, IV
2DE-proteomics coupled with LC/MS	II
Quantitative analysis of toxins by LC/MS	I–IV
Chlorophyll A measurement	I–IV
Determination of enzymatic activity	IV
Methane analysis by gas chromatography (GC)	IV
Bioinformatics	
Galaxy interface	I, II, IV
Phylogenetic reconstruction	I, II
Genome annotation	I, II, IV

4 Results and discussion

Genomes of toxic and bloom-forming brackish water cyanobacteria (I, II)

Whole genomes of the Baltic Sea cyanobacteria, *N. spumigena* UHCC 0039 (I) and *Dolichospermum* sp. UHCC 0315 (II), were sequenced in this study to increase the information concerning the metabolic capacities and the number of high-quality reference genomes of brackish water cyanobacteria. Despite intense cyanobacterial genome sequencing during the past ten years, the available genomic information is still rather skewed, focusing mainly on the draft genomes of picocyanobacteria *Prochlorococcus* and *Synechococcus* (Shih *et al.*, 2013). This is partially due to the methodological challenges of assembling cyanobacterial genomes containing a great deal of repetitive elements. The total number of genome sequences among brackish water cyanobacteria is small despite their high abundance and important role in ecosystems. The paucity of available genomes has also posed a challenge for the assembly of brackish water metagenomic data (Celepli *et al.*, 2016).

In Bergey's Manual of Systematic Bacteriology, taxonomical classification of prokaryotes is based on 16S ribosomal RNA (16S rRNA) sequences together with morphology and the DNA-DNA hybridization technique (Castenholz, 2001). This classification divides the cyanobacteria clade into five subsections, of which subsection IV comprises all heterocystous *Nostocales* species (Castenholz, 2001) including *N. spumigena* UHCC 0039 and *Dolichospermum* UHCC 0315. However, a genome-scale comparison adds more value and accuracy to the previously accepted classification of genetic and evolutionary relatedness. Within *Nostocales*, seven subclades (Ia–d; II a–c) were distinguished in this study based on phylogenomic analysis of 31 conserved proteins (Figure 4a). Branches were further identified from average nucleotide (ANI) (Konstantinidis and Tiedje 2005a) and amino acid identity (AAI) (Konstantinidis and Tiedje 2005b) analyses (4b–c). All three *N. spumigena* strains for which genomes are available, UHCC 0039, CCY 9414, and CENA 563, branched to one subcluster (Id), whereas the clustering of other species in the *Nostocales* order was more diverse. The genus *Anabaena* was reorganized during this study, and new nomenclature has slowly been adopted in its classification. In the new nomenclature, *Dolichospermum* represents planktonic species, whereas *Anabaena* comprises benthic species (Wacklin *et al.*, 2009). However, taxonomy between these two genera and even *Aphanizomenon* and *Nostoc* species is still not well defined (Halinen *et al.*, 2007; 2008), which may partially explain the incoherence in clustering.

N. spumigena UHCC 0039 showed high similarity to previously sequenced isolates from the Baltic Sea (CCY 9414) (Voß *et al.*, 2013) and a shrimp pond

in Brazil (CENA 563) (Popin *et al.*, 2016) at the nucleotide and amino acid levels, which indicate their short evolutionary distance and common origin (Figure 3). Furthermore, pangenome analysis showed that 4375 out of 5439 identified proteins from all sequenced *N. spumigena* strains were shared by at least two strains and only 278 orphan clusters were identified from the genome of *N. spumigena* UHCC 0039 (Figure 3). Most of the orphan clusters represented less studied short proteins with <80 amino acids (μ -proteins), the role of which is mostly unknown, but their function as regulatory elements has deserved particular attention (Baumgartner *et al.*, 2016). In addition to μ -proteins, a unique set of σ factors from the genome of *N. spumigena* UHCC 0039 was found, one of which lacked the homologs from the model *Synechococcus* and another one from other sequenced *N. spumigena*. Sigma factors have been established to be ubiquitously important for niche adaptation and regulation of gene expression, and a set of alternative sigma factors identified from the *N. spumigena* UHCC 0039 genome thus indicates adaptation to the brackish water environment.

Another sequenced cyanobacterium from the Baltic Sea, *Dolichospermum* UHCC 0315, showed high synteny to the freshwater strain *Dolichospermum* UHCC 0090 (Wang *et al.*, 2012), implying that UHCC 0315 has a freshwater origin. Interestingly genome closure revealed two substrains of *Dolichospermum* UHCC 0315 (A and B). Substrain A, which has five additional genes, was buoyant whereas substrain B grew on the bottom of the cultivation flask despite both substrains carrying a full set of gas vesicle gene cluster (*gvp*) genes (genes that are known to participate in gas vacuole formation similarly to *Nostoc* PCC 7120; Kaneko *et al.*, 2001). Closer analysis of the five additional genes found in substrain A tentatively points towards the modification of surface proteins and might thus be linked to buoyancy. The identification of two substrains in the same culture underlines the importance of whole genome analysis to elucidate genetic structures and enhance knowledge about novel genetic elements. The method coupling short-read Illumina and long-read Pacific Biosciences (PacBio) sequences used in this study provides a good platform for sequencing plastic genomes with a high number of repetitive elements.

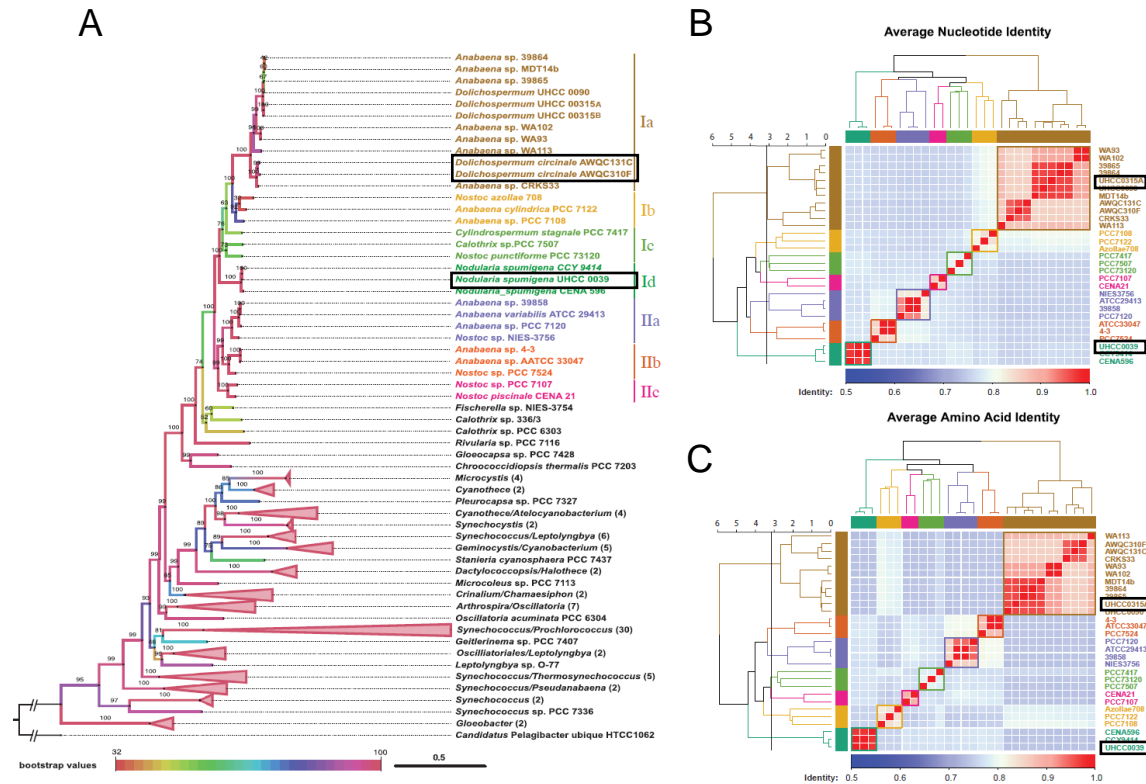


Figure 3 Maximum likelihood phylogenomic tree of sequenced cyanobacteria based on the concatenated alignment of 31 universal marker genes (Wu and Eisen, 2008) (a), average nucleotide identity (b), and average amino acid identity (c). Clustering of the average nucleotide identity (ANI) and average amino acid identity (AAI) was performed using average linkage hierarchical clustering based on pairwise Euclidean distances, and subgroups Ia–d, IIa–c were determined by cutting the dendrograms at a tree height of 3.2.

Distribution and transcriptional adaptation of toxic cyanobacterial species in different salinities (I, II)

Dolichospermum sp. needs freshwater conditions for proper proliferation (Brutemark *et al.*, 2016), whereas *N. spumigena* is generally perceived as a brackish water species. However, these two toxic genera coexist in the cyanobacterial blooms of the Baltic Sea (Stal *et al.*, 2003). *N. spumigena* dominates in the more saline open-sea and *Dolichospermum* in coastal regions, which raises the question of their potential to adapt to different salinities that occur in the Gulf of Finland and coastal Baltic Sea. In this study, based on comparative genomics, the Baltic Sea strain *Dolichospermum* UHCC 0315 had high synteny with the previously sequenced freshwater strain *Dolichospermum* UHCC 0090 (Wang *et al.*, 2012), leading to the conclusion that this cyanobacterium has a freshwater origin. Growth of *Dolichospermum* sp. UHCC 00315 was strongly arrested at a salinity of 6 g NaCl L⁻¹ while *N. spumigena* UHCC 0039 was unable to proliferate in freshwater conditions (0 g L⁻¹ NaCl). In turn, *Dolichospermum* sp. UHCC 0315 grew fastest in 0 g L⁻¹ NaCl implying that the abundance and distribution of *Dolichospermum* sp. will increase in the future, as climate change drives a decrease in salinity.

At the molecular level, possession of compatible solutes is the most important long-term salt acclimation mechanism of cyanobacteria (Hagemann, 2011; Klähn and Hagemann, 2011). These organic sugars protect cellular structures and functioning from an increased amount of Na⁺ ions. Sucrose and trehalose are the most commonly found compatible solutes in fresh- and brackish water cyanobacteria (Klähn and Hagemann, 2011). The genes for sucrose synthesis were found from both the *N. spumigena* UHCC 0039 and *Dolichospermum* sp. UHCC 0315 strains, whereas the genes for trehalose synthesis were only found from *N. spumigena* UHCC 0039. Increased expression of the genes related to compatible solute synthesis was not observed in any of the studied salinities investigated in this study. In line with this, although metagenomic data showed an increased abundance of trehalose synthesis genes at higher salinities in the Baltic Sea, neither transcripts nor trehalose were detected, suggesting the silence of this genetic region (Celepli *et al.*, 2016). These results call into question the importance of trehalose for Baltic Sea *N. spumigena* cyanobacteria, despite a clear relationship between higher salinities and an increased frequency of trehalose synthesis genes.

Several studies have shown that molecular changes in cyanobacteria acclimating to high salt concentrations occur within an hour (Al-Hosani *et al.*, 2015; Billis *et al.*, 2014; Qiao *et al.*, 2013; Marin *et al.*, 2004), but long-term acclimation and cyanobacterial responses to unfavorably low salinity (hypotonic environment) has not received that much attention. In the cyanobacteria studied here, both unfavorably low and high salinities induced general stress responses, including reconstruction of transcripts related to the photosynthetic apparatus,

chaperones, and transcription and translation. Unfavorably high salinity strongly reduced the expression of genes related to nitrogen fixation in *Dolichospermum* UHCC 00315, which was detected as a demand for nitrate reduction, circulation of amino acids, and fine-tuning of amino acid biosynthesis. In addition, as already shown previously (Latifi *et al.*, 2009; He *et al.*, 2002), unfavorably high salinity triggered oxidative stress, which was seen as induction of genes related to fatty acid synthesis and lipid metabolism, i.e. molecules that are the easily damaged by peroxides. A salt-free environment remodeled the expression of 6 σ factors in *N. spumigena* UHCC 0039, demonstrating the importance of its unique pool of σ factors for brackish water adaptation.

Strategies of toxic and diazotrophic cyanobacteria for coping with limitation of inorganic phosphorus (III, IV)

Cyanobacteria encountering a broad range of environmental conditions have been found to possess a larger genome size compared to those living in more stable environments (Biller *et al.*, 2015). In the Baltic Sea, where cyanobacteria are exposed to phosphorus limitation, they expectedly carry a wide combination of the genes in the *pho* regulon, which allows them to exploit several different phosphorus sources during P_i scarcity. Due to the importance of phosphorus, it was unsurprising that the studied cyanobacterial strains, *Dolichospermum* UHCC 0090 (closely related to *Dolichospermum* UHCC 0315) and *N. spumigena* UHCC 0039, were unable to proliferate in conditions where all phosphorus sources were omitted. A lack of phosphorus hampered cellular growth and metabolism along with the induction of alkaline phosphatase activity. Both studied cyanobacterial strains harbor a high affinity *pstABCS* transporter system, which became highly induced under P_i scarcity and exhibited an important role in efficient P_i uptake.

In this study, *N. spumigena* UHCC 0039 was found to carry the *phn* gene cluster, similar to *N. spumigena* CCY 9414 (Voß *et al.*, 2013), implying that *N. spumigena* may be capable of transporting and utilizing phosphonates. Genome mining and PCR studies showed that the *phn* gene cluster is highly conserved among *N. spumigena* cyanobacteria (Figure 4A–C), whereas only 5.4% of all 500 sequenced cyanobacteria (April, 2017) carry the *phn* gene cluster. Using *N. spumigena* specific primers, the genes *phnJ* and *phnD* were also found from environmental samples from the Baltic Sea, revealing their wide distribution in this area (Figure 4D). The *N. spumigena* strains UHCC 0039 and UHCC 0060 were capable of utilizing the small and naturally produced phosphonates, methylphosphonate (MPn), ethylphosphonate (EPn), and 2-Aminoethylphosphonate (2APn), which enabled them to grow in P_i -free medium supplemented only by phosphonates. Growth in the MPn medium simultaneously freed methane into the gaseous environment, and thus massive cyanobacterial

blooms may contribute to methane supersaturation in the Baltic Sea in a similar fashion as described for the oceans (Karl *et al.*, 2008; del Valle and Karl, 2014; Repeta *et al.*, 2016). RNA-seq data showed only minor transcriptional reconstruction, demonstrating high suitability of MPn for *N. spumigena*. These results suggest that the capacity to utilize phosphonates produces a competitive advantage for *N. spumigena* over *Dolichospermum* sp. and *Aphanizomenon flos-aquae* in P_i -limited blooms in the Baltic Sea. This could also explain their higher abundance in the open-sea regions of the Baltic Sea, where the waterbody is not as highly influenced by mainland P_i runoffs as in coastal regions.

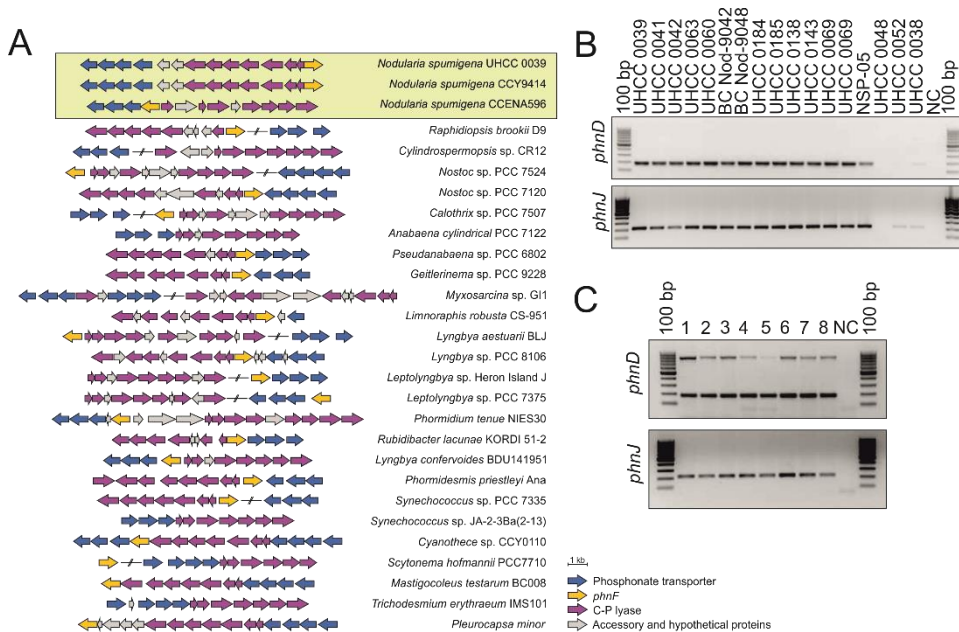


Figure 4 Structures of *phn* gene clusters in cyanobacteria. A genome mining study showed that only 5.4% of sequenced cyanobacteria carry the *phn* gene cluster (a). The *phn* gene cluster identified in the genome of *N. spumigena* UHCC 0039 (b). Based on the PCR study using *N. spumigena* specific primers, all *N. spumigena* strains harbor the *phn* gene cluster (c) and the gene cluster is also widely distributed along the Baltic Sea (D).

Link between environmental conditions and capacity of cyanobacteria to produce bioactive compounds (I–IV)

The genomes of *Dolichospermum* UHCC 0090 and 0315 harbor gene clusters for microcystin biosynthesis (microcystin synthetase gene cluster, *mcy*), whereas *N. spumigena* UHCC 0039 has genes involved in nodularin biosynthesis (nodularin synthetase gene cluster, *nda*). Furthermore, studied strains possess gene clusters of anabaenopeptin, anabaenopeptilide, hassallidin, anacyclamide, spumigin, nodulapeptin, and heterocyst glycolipid but also several gene clusters

participating in the synthesis of unknown products. Gene clusters with known products are presented in Figure 5. The structures and biosynthesis of these cyclic peptides have been described previously (Botes *et al.*, 1985; Rinehart *et al.*, 1988; Fujii *et al.*, 1997; Sivonen and Börner, 2008; Leikoski *et al.*, 2010; Fewer, 2013).

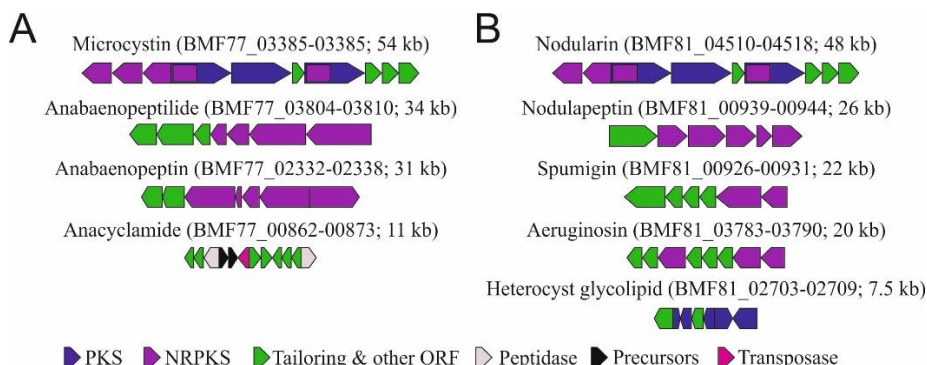


Figure 5 Gene clusters for bioactive peptides produced by *Dolichospermum* UHCC 0315 (a) and *N. spumigena* UHCC 0039 (b). Gene clusters were originally published earlier (Botes *et al.*, 1985; Rinehart *et al.*, 1988; Fujii *et al.*, 1997; Sivonen and Börner, 2008; Leikoski *et al.*, 2010; Fewer, 2013).

Favorable environmental conditions have frequently been found to enhance the synthesis of toxins and other bioactive products (Rapala *et al.*, 1997; Repka *et al.*, 2004; Harke and Gobler, 2013). However, the intracellular concentration of microcystin in *Dolichospermum* sp. increased at unfavorably high salinity (Brutemark *et al.*, 2015). Nevertheless, predicting the concentration of bioactive peptides in various environments is difficult because the indigenous role of these molecules is mainly unknown. In this study, high salinity increased the production of microcystins, similar to Brutemark *et al.* (2015), whereas P_i starvation induced the *mcy* gene cluster although differences in microcystin concentration were not found. Microcystin is suggested to serve as a protective element against oxidative stress in cyanobacteria (Dziallas and Grossart, 2011; Zilliges *et al.*, 2011) and results reported here support this as high salinity may trigger oxidative stress conditions in the cell (He *et al.*, 2002; Latifi *et al.*, 2009).

On the contrary, unfavorable salinity, low P_i , and phosphonate treatment seemed to have no effect on nodularin production or transcription of the *nda* gene cluster. However, transcription of the cryptic gene cluster (BMF81_00493-00562) identified from the genome of *N. spumigena* UHCC 0039 was strongly affected by changing environmental conditions, experiencing upregulation in the MPn medium and downregulation in unfavorably low salinity. The gene cluster has great similarity to that found in the genome of *Agrobacterium tumefaciens* (Rondon *et al.*, 2004) and *Nostoc* sp. PCC 7120 (Jeanjean *et al.*, 2008), suggesting its role in siderophore biosynthesis. The product of this cryptic gene cluster remains unclear.

In conclusion, varying environmental conditions differentially affected the capacity of cyanobacteria capacity to synthesize bioactive products, and correlations were not found between mRNA frequency and the concentration of certain peptides. The amount of transcripts should thus not be used as a proxy for toxin concentration in the waterbody.

Responses of cyanobacteria to varying environments (I–IV)

Cyanobacteria can adapt rapidly to a wide variety of geochemical and climatic changes by inducing or repressing the expression of various parts of their genomes (Adams *et al.*, 2008; Harke and Gobler, 2013; Qiao *et al.*, 2013; Kopf *et al.*, 2014; 2015). The reconstruction of metabolism usually causes chlorosis and hinders growth in unfavorable conditions, which can be detected as lowered optical density, even by the naked eye. To maintain vitality under the experimental conditions, cyanobacteria fine-tuned several of their key metabolic processes at the mRNA level (Figure 6). Unfavorable salinity and phosphorus limitation appeared to conspicuously arrest expression of the genes in the photosynthetic apparatus. In addition, demand of the alternative protein pool was clearly seen as the circulation of amino acids along with changing the expression pattern of the transcription and translation apparatus, including the σ factors. Interestingly, despite P_i being the most preferable form of phosphorus for cyanobacteria, the transcriptome profile of the culture grown in the MPn medium was very similar to that grown in the normal culture medium containing P_i .

Changing environmental conditions, namely unfavorable salinity and P_i limitation, additionally induced the expression of several genes producing hypothetical and unknown proteins. These proteins are widely distributed among cyanobacterial genomes and represent the incomplete state of knowledge concerning the metabolism and molecular structure of cyanobacteria. As an example, a giant protein of 1896 amino acid residues containing a phytase-like domain was heavily upregulated under phosphorus limitation, suggesting an important role in the phosphorus metabolism of *Dolichospermum* UHCC 0090. Hypothetical proteins were not given a great deal of attention in this work, because discovering their function is another large field of research. However, comparative transcriptomics can provide a starting point for unraveling the reservoir of this uncharacterized pool of proteins and in helping to find new metabolic processes in cyanobacteria.

Transcriptomes are widely used to indicate cellular status in stress conditions, but the method yields information only from the first level of the regulatory network. Furthermore, transcriptomes have been found to correlate poorly with proteomes (Wasinger *et al.*, 2006). Thus, the study of proteomics was included to better understand the cellular acclimation processes of *Dolichospermum* UHCC 0090

under P_i scarcity. 2-DE proteomics revealed only 43 differentially expressed proteins, which mainly represent central metabolism. Due to methodological challenges in obtaining sufficient sensitivity, the applied proteomics study was not found to produce information complementing the RNA sequencing data and was thus not used more widely in this study.

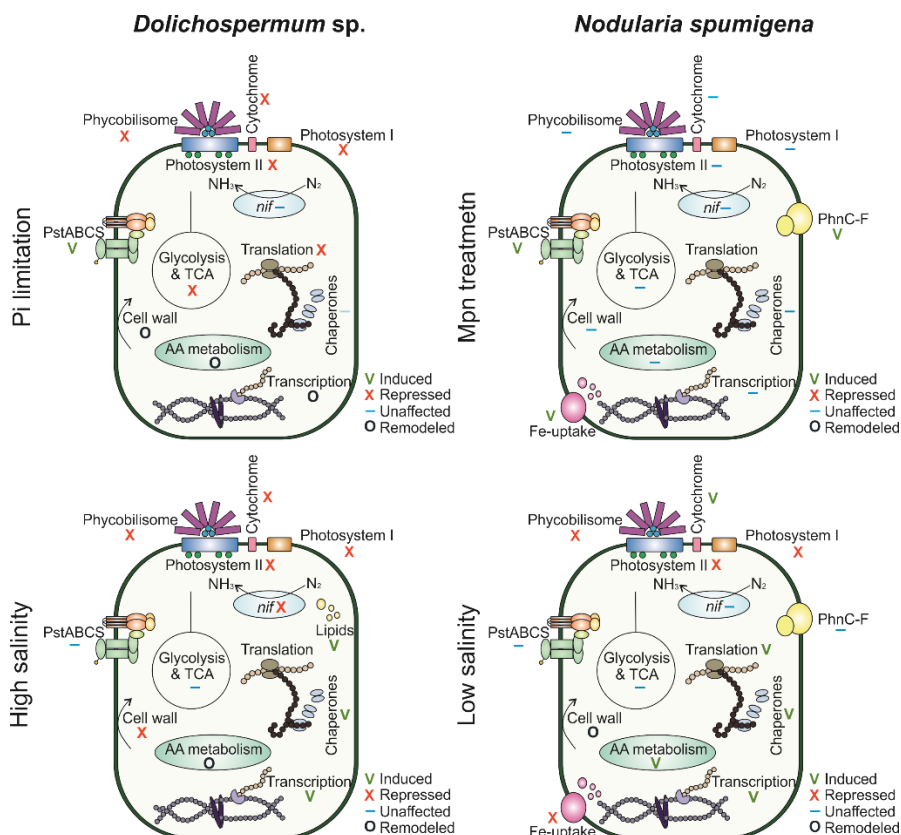


Figure 6 Responses in key metabolic processes at the mRNA level in *Dolichospermum* sp. and *N. spumigena* in the studied environments.

Identification of environmental markers to assess the status of cyanobacterial blooms (I–IV)

Despite its high sensitivity to contemporary environmental conditions along with its importance in nutrient circulation and flux, bacterioplankton is only marginally included in the Baltic Sea monitoring program (HELCOM 2012a, b). The determination of key genes and their differential expression in varying environments (marker genes) may, however, substantially improve and complement chemical analyses. However, limited knowledge, and expensive and

time-consuming methodologies have obstructed their adoption into monitoring programs. Recently, high-throughput DNA sequencing has helped to overcome these challenges by enabling the identification of a wide set of putative marker genes and simultaneously improving general knowledge about the metabolism and niche adaptation strategies of organisms.

This study did not identify any stress-specific markers against long-term exposure to unfavorable salinities. The genome of *N. spumigena* UHCC 0039 encodes genes participating in sucrose and trehalose synthesis, whereas *Dolichospermum* UHCC 0315 only encodes the former, but neither of these genes were differentially expressed. Previously, the role of trehalose as a compatible solute in diazotrophic cyanobacteria has been questioned (Higo *et al.*, 2006; Möke *et al.*, 2013; Celepli *et al.*, 2017), despite the fact that the frequency of this gene has appeared to increase with increased salinity in the Baltic Sea (Celepli *et al.*, 2017). Regardless of whether trehalose has a role in salt acclimation, our results support previous findings showing that the trehalose synthase gene cluster is more abundant at higher salinity.

It is generally agreed that Baltic Sea cyanobacterial blooms are often phosphorus-limited (Nausch *et al.*, 2004; Olofsson *et al.*, 2016), but the situation is complicated by the chemistry of phosphorus and the capacity of cyanobacteria to contribute to phosphorus circulation between various oxidation states (Dyhrman *et al.*, 2006; Adams *et al.*, 2008). An increased activity of alkaline phosphatase is commonly used to indicate phosphorus scarcity in bacteria and aquatic ecosystems (Li *et al.*, 1998; Nausch, 1998; Van Wambeke *et al.*, 2002), but APA activity and P_i load were not found to be correlated in the southern Baltic Sea (Schaub, 2017, personal communication). In this study, alkaline phosphatase activity remained low when another suitable phosphorus source was available (here, MPn). Based on this, APA is suitable as a marker for the scarcity of P_i but not for phosphorus as a whole, and an additional molecular marker should be included for monitoring phosphorus status in cyanobacterial blooms.

P_i scarcity is expected to induce all genes located under the *pho* regulon, but this was not found in the present study. In addition, it is not fully clear what the other genes are that become upregulated under P_i scarcity (Su *et al.*, 2007). In the study of diazotrophic *Crocosphaera watsonii*, Pereira *et al.*, (2016) suggested that a single genetic marker is not enough to characterize phosphorus stress in cyanobacteria, but a combination of *pstS* and the arsenite efflux protein, *arsB*, gave desirable results. In this study, expression of the *pstS* gene heavily increased when P_i was omitted, but, possibly due to the insufficient sensitivity of the RT-qPCR method used, some discrepancy was also found. The phosphonate transporter gene *phnD* was another constantly expressed gene in P_i -limited conditions. However, the *phnD* gene is not found in all cyanobacterial genomes, although it is much more widely distributed compared to the phosphonate lyase complex (*phnH-M*), and thus its suitability as a marker is limited. In addition, this study showed that *phnJ* was constantly expressed only in the presence of a

suitable substrate, namely phosphonates, and the gene is therefore a promising indicator gene for reporting the bioavailability of phosphonates for cyanobacteria. The determination of phosphonate concentration by chemical analyses is challenging, and the analysis of gene expression in environmental samples could complement chemical analyses and aid in the identification of phosphonates in the Baltic Sea. This study demonstrates that using a combination of several approaches, both chemical and biological, for monitoring phytoplankton communities can improve the accuracy of the information obtained regarding this incompletely understood link of the nutrient cycle.

Implications of results in wider ecological perspective (I–IV)

Despite the key focus of the current study being in the ecology of cyanobacteria in the Baltic Sea, the results also have implications beyond the Baltic Sea due to similarities in growth conditions between aquatic ecosystems. The comparative genomics analysis in this study showed that the genomes of all the sequenced *N. spumigena* strains are highly similar. Moreover, high synteny between brackish and freshwater *Dolichospermum* sp. was found and genomic similarities indicate that the studied cyanobacteria have similar within-genus adaptation strategies globally. The experimental studies further showed that *Dolichospermum* sp. is a freshwater genus tolerating only minor increases in salinity whereas the blooms of *N. spumigena* always require salt in the growth medium.

This study indicates that cyanobacterial strains with higher salt tolerance might have more complex phosphorus scavenging capacities than strains originating from freshwater. Identifying potential associations between salt tolerance and the evolution of complex phosphorus metabolism is beyond the scope of this work. However, this would make sense in light of the phosphorus distribution in aquatic ecosystems: elevated P_i concentrations in eutrophic freshwater ecosystems and estuaries usually result from human activities (Conley *et al.*, 2009), whereas marine systems are oligotrophic with very low availability of P_i and other nutrients, and higher cellular investment is needed to gain an adequate amount of nutrients (Van Wambeke *et al.*, 2008). In addition, this study shows that the phosphonate degradation pathway based on the *phn* gene cluster is highly conserved among *N. spumigena* (Figure 7), whereas the pathway is missing from the freshwater species *Dolichospermum* sp. This phenomenon is also well characterized in marine ecosystems (Dyhrman *et al.*, 2006; 2009, Repeta *et al.*, 2016), and together these results suggest that more complex phosphorus metabolism is needed in areas with low levels of P_i inflow. However, the concentration of anthropogenic phosphonates may be higher in freshwater ecosystems compared to the open sea, and other currently unknown phosphonate degrading pathways besides the *phn* gene cluster can also be possible. Evidence tentatively supporting this hypothesis has already been found in the study of *Anabaena variabilis* ATCC 29413 (Drzyzga *et al.*, 2017).

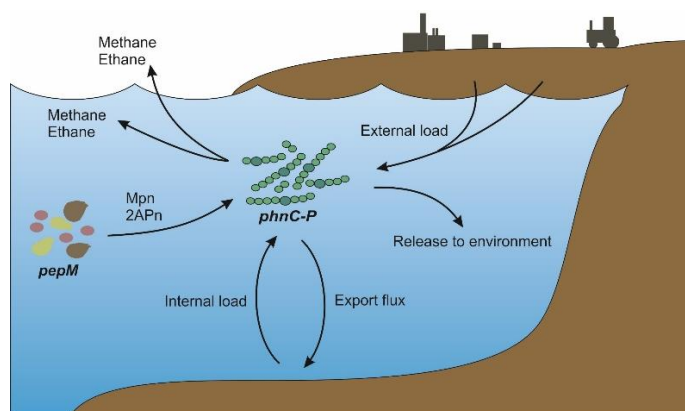


Figure 7 Schematic illustration of the phosphonate cycle in the Baltic Sea. Organisms harboring the *pepM* gene release methylphosphonate or 2-aminoethylphosphonate (among other organophosphates) into the water, which can be utilized by (cyano)bacteria harboring the *phnC-P* gene cluster. An organic remnant (e.g. methane or ethane) is released to the environment, whereas inorganic phosphorus is used by the organism. Mpn = Methylphosphonate, 2APn = 2-aminoethylphosphonate, *pepM* = phosphoenolpyruvate phosphomutase; *phnC-P* = phosphonate gene cluster.

Notably, experimental approaches always have limitations. The cyanobacterial strains in the HAMBI/UHCC culture collection have been maintained in liquid, nutrient-rich and nitrogen-free culture medium (Z8X) under continuous illumination for 20–30 years (e.g. Sivonen *et al.*, 1989; Halinen *et al.*, 2007). Loss of buoyancy as described in this study is only one example of the ongoing evolution of the strains deposited in the culture collection. Deletion of accessory functions that are unnecessary in stable and nutrient-replete laboratory conditions has also been reported elsewhere (Wang *et al.*, 2012). In addition, experimental conditions always differ from growth conditions in the nature. In the context of this study, major differences include nutrient-replete conditions, lack of mixing pressure, and low light intensity. However, despite the fact that experimental findings based on one or two bacterial isolates should not be generalized to the nature, laboratory experiments provide valuable knowledge about biological systems and facilitate generation of hypotheses that can later be tested in field studies.

5 Final remarks and future considerations

Two newly sequenced Baltic Sea cyanobacteria, *N. spumigena* UHCC 0039 and *Dolichospermum* sp. UHCC 0315, showed high synteny with their previously sequenced relatives. However, in a comparative genomic analysis, *Dolichospermum* sp. branched into several phylogenetic subclusters, implying a discrepancy in the taxonomy of the genus. Subclusters were likely a consequence of misidentification of species based on morphology along with inconsistent nomenclature. Close synteny between *Dolichospermum* UHCC 0315 and a freshwater *Dolichospermum* strain showed that Baltic Sea *Dolichospermum* has a freshwater origin, but the genus has also been transported to the brackish water environment over the course of evolution. This was further supported by the harmfulness of moderate salinity to the growth of *Dolichospermum* and the induction of a general stress response in the cells. Interestingly, the genome closure of *Dolichospermum* sp. UHCC 0315 resulted in distinguishing two subtypes, A and B, of which subtype A carried five extra genes and had a buoyant phenotype. This shows that draft genome sequences can fail to identify important genetic structures, and whole genome sequencing should therefore be preferred. The state-of-the-art hybrid Illumina-SMRT sequencing technique used in this study was found to be a robust method for improving the sequencing accuracy of cyanobacterial genomes that carry a high number of repetitive elements.

Whereas Baltic Sea *Dolichospermum* sp. was found to have a freshwater origin, *N. spumigena* tolerates a much higher concentration of salt, giving it a competitive advantage in more saline regions of the Baltic Sea. Pronounced genetic synteny showed by comparative genomic analysis indicates a short evolutionary history of Baltic Sea *N. spumigena* strains. In addition to a high number of insertion sequences and CRISPR-Cas cassettes, a unique set of sigma factors indicate remarkable plasticity of *N. spumigena* during salt acclimation. Salinity appeared to have a high impact on the species distribution of *Dolichospermum* and *N. spumigena*. In the presence of climate change and a predicted decrease in salinity, *Dolichospermum* blooms may outcompete *N. spumigena* in wider regions and shift southward.

Another important abiotic factor controlling diazotrophic cyanobacterial blooms in the Baltic Sea is phosphorus availability. Both *N. spumigena* and *Dolichospermum* sp. harbor *pstABCS* transporter systems in their genomes, which became highly upregulated under P_i limitation. In addition, *N. spumigena* was found to be able to utilize naturally produced phosphonates, mainly methylphosphonate, as an alternative phosphorus source. This characteristic not only has the potential to make *N. spumigena* an important contributor in the phosphorus cycle in the Baltic Sea, but also serves as a competitive advantage in P_i -limited cyanobacterial blooms. Utilization of methylphosphonate released the

greenhouse gas methane into the gaseous environment, indicating a contribution by *N. spumigena* blooms to aerobic methane release. The phosphonate concentration in the Baltic Sea is unknown, but this particular form of phosphorus should not be ruled out when devising strategies to control cyanobacterial blooms in the Baltic Sea.

Nutrient deficiency and varying salinity are only two among several environmental stressors that cyanobacteria encounter in aquatic ecosystems. Moreover, importantly, cyanobacteria always co-occur with other organisms in the environment, interacting closely with grazers, heterotrophic bacteria, and viruses. Modeling multi-level systems and extreme environments is highly challenging in experimental conditions, because all microbes cannot be cultivated in the laboratory. Recent developments in culture-independent methods, including metagenomics, -transcriptomics and -proteomics, as well as metabolomics, have helped to overcome this bias, and single-cell approaches, in turn, facilitate the identification of less abundant or completely uncharacterized species to gain an even deeper understanding of microbial life in water ecosystems. Therefore, although comprehensive disentangling of the complexity of multi-level networks and metabolism in harsh environments is still far from our reach, the near future holds the promise of taking our understanding to an unprecedented level of sophistication.

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